# Cross-talk between intra-ovarian factors in zebrafish: potential influence of endocrine disrupting chemicals

**Research proposal**
**(To be pursued during Ph.D.)**

**Synopsis submitted by**

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## Introduction

The ever increasing demand for fish has ignited endocrinologists and reproductive biologists to unravel the string of complex yet highly organized and systematically designed cascades regulating ovarian functions. In fish and in other vertebrates, ovarian development and function is controlled by pituitary gonadotropins (GTHs), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), that act on follicular theca-granulosa cells surrounding growing oocytes and promote synthesis of two major sex steroids namely, 17β-estradiol ($E_2$) and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P), respectively (reviewed in Nagahama, 1994). Fish oocytes arrested at first meiotic prophase, undergo a massive growth (vitellogenesis) under the regulation of $E_2$ (Specker and Sullivan, 1994), followed by resumption of cell cycle via 17,20β-P, the maturation inducing steroid (MIS). Earlier evidences in zebrafish (*Danio rerio*) ovary have established that binding of MIS to membrane progestin receptor-α (mPRα) at the oocyte surface promotes activation of maturation promoting factor (MPF), a hetero-dimer of cdc2 and *de novo* synthesized cyclin B (Zhu et al., 2003; reviewed in Nagahama and Yamashita, 2008). Activation of MPF leads to withdrawal of prophase (G2) arrest and resumption of meiotic maturation through histone H1 kinase activation, phosphorylation of nuclear lamins, germinal vesicle breakdown and finally first polar body exclusion to form fertilizable (haploid) female gametes (reviewed in Nagahama and Yamashita, 2008). Interestingly, immature oocytes at prophase I possess high cyclic-AMP, either from surrounding follicular layers through the gap junctions or by activation of $G\alpha_s$-mediated adenylyl cyclase (AC) upon $E_2$ binding to a novel G-protein estrogen receptor (GPER) on the oocyte surface (reviewed in Das et al., 2017; Nagahama and Yamashita, 2008). Conversely, 17,20β-P binding to mPRα activates $G_{\alpha i}$, which in turn attenuates AC activity and prevents cAMP production thereby driving the oocyte towards final maturation (Zhu et al., 2003; Pang and Thomas, 2010).

Additionally, an increasing body of evidence suggests that the actions of gonadotropins are modulated by a variety of locally produced non-steroidal peptide factors viz. insulin-like growth factors (IGFs) (Reinecke, 2010; Li et al., 2015; Das et al., 2016), epidermal growth factor (EGF) family (Panigone et al., 2008; Su et al., 2010; Chung and Ge, 2012; 2013), TGFβ superfamily (Juengel et al., 2004; Moore and Shimasaki, 2005) control ovarian functions. In zebrafish ovary, differential expression of these autocrine/paracrine factors and their cognate receptors, either at follicular theca-granulosa cells or oocyte itself, at various stages of oogenesis, are believed to form an intimate regulatory bidirectional communication between both the compartments and has been an area of active research in recent past (reviewed in Albertini et al. 2001; Ge, 2005; Biswas and Maitra, 2017).

Increasing public concerns over the adverse effects of anthropogenic contaminants, especially endocrine disruptors, on the health and well-being of both humans and wildlife is a hot debate nowadays. In addition to regulation by the hypothalamo-pituitary-gonadal axis, anthropogenic contaminants, especially estrogen mimics, have been shown to modulate various physiological processes adversely affecting gonadal growth and development in non-target organisms. EDCs may exert their effects by mimicking the behavior of endogenous

hormones, specifically through estrogenic or androgenic modes of action, thereby perturbing the natural course of receptor binding and action. Fish are considered as one of the primary risk organisms for EDCs as they are directly exposed to a wide variety of EDCs, originating from industrial, agricultural or municipal effluents, in their aquatic environment. Plasticizers like bisphenol A (BPA), nonylphenol (NP), surfactants, detergents, pesticides and other aromatic compounds from industrial effluents have been shown to produce negative, long-term effects on reproduction (Kidd et al., 2007) in particular, the quality of the developing ovarian follicle and oocyte (Pocar et al., 2003) in diverse fish species. However, influence of EDCs on ovarian stress response, GTH action and expression of steroidogenic enzyme genes vis-a-vis expression of ovarian growth factor system have not been studied in great detail yet in the ovary of zebrafish, a species of huge importance as one of the most widely used model organisms in recent times.

**Origin of the problem**

Studies in zebrafish ovary as well as in mammals indicate that biosynthesis of gonadal steroids as well as juxtacrine peptide factors are subjected to regulation by pituitary-derived GTHs (Park et al., 2004; Li et al., 2015). Besides these regulatory networks, both steroid- and peptide-mediated, are believed to have pivotal influence in controlling each ovarian function which includes folliculogenesis and development of maturational competence at the cellular level. However, **potential cross-talk between steroids- and growth-factor-mediated signalling cascades as well as potential influence of endocrine disruptors (BPA, NP) on such processes in *D. rerio* ovary have not been fully explored yet and require further investigation**. Accordingly, the present proposal seeks to explore the participation of molecular candidates in the bi-directional communication between the intra-ovarian compartments and potential influence of estrogen mimicking endocrine disruptors (BPA, NP) taking zebrafish as the model organism.

**Objectives:**

Based on the above observation the following objectives have been proposed:

1. **To study the molecular mechanisms underlying gonadotropin regulation of follicular growth, shift in steroidogenesis and attainment of maturation competence, primarily through transcript abundance of GTHR, GPER and mPRα, in zebrafish ovary.**

2. **Potential cross-talk between GTH/IGF/EGF-mediated signalling events in any or all of the above processes.**

3. **Profiling expression of steroidogenic enzyme genes and autocrine/paracrine factors and its possible impact on ovarian growth, oocyte quality and steroid levels in bisphenol A/ nonylphenol exposed female zebrafish.**

## Methodology

### Animal and Oocyte Culture

Adult zebrafish will be collected from fish farms in and around Santiniketan, West Bengal, India, and will be maintained in 30L glass aquaria, at 28±1 ºC on a 14L:10D photoperiod. Fish with gravid ovary will be dissected to collect full-grown, immature ovarian follicles in ice-cold $Ca^{2+}$ free zebrafish Ringer (116mM NaCl/ 2.9mM KCl/ 5Mm HEPES, pH-7.2) (Kondo et al., 1997) and will be manually separated and washed several times with the zebrafish Ringer with 0.6% salinity supplemented with penicillin (100U/ml) and streptomycin (100µg/ml). Healthy oocytes will be selected after 1h pre-incubation prior to the addition of hormones and/or inhibitors. Viability of oocytes will be ascertained by Trypan blue dye exclusion method. Activators for AC (forskolin), MAPK (okadaic acid) or inhibitors for AC (SQ22356), PKA (H89/PKI-amide), PI3K (wortmannin/LY294002), Akt/PKB (SC 66/10-DEBC hydrochloride), PDE (IBMX, cilostmide, rolipram), MEK (U0126/PD098,059), steroidogenic enzymes (DL-Aminoglutethimide/Trilostane) and cyclohexamide (translational inhibitor), actinomycin D (transcriptional inhibitor), either in combination or alone, will be used 1h prior to the addition of any exogenous hormone or growth factor. Concentration of these inhibitors for *in vitro* experiments will be chosen after testing dose response and viability with Trypan blue.

### Determination of GVBD

Migration of Germinal Vesicle (GV) towards the periphery of the oocyte and GVBD will be determined by placing post-incubated oocytes in clearing solution (ethanol, formaldehyde and acetic acid in a ratio 3:6:1). (Lessman and Kavumpurath, 1984)

### Enzyme-Linked Immunosorbant Assay (ELISA)

Media from zebrafish follicle incubations will be collected, lyophilized, reconstituted and measured for steroid levels (Testosterone, $E_2$, 17,20β-P) by enzyme immunoassay (EIA) kit following manufacturer's protocol.

### Preparation of oocyte extracts

100 follicle-enclosed oocytes will be used per incubation. Oocytes will be washed thrice with extraction buffer (EB) containing 100mM sodium β-glycerophosphate, 20mM HEPES, 15mM $MgCl_2$, 5mM EGTA, 100µM PMSF, 3µg/ml leupeptin, 10µg/ml aprotinin, pH-7.5 (Hirai et al., 1992). After removing excess EB with filter paper, 100µl of fresh ice-cold EB will be added and the oocytes will be homogenized in a glass homogeneizer. The homogenate will be centrifuged at 12,500 X g for 30 mins at 4 ºC and the clear cytosolic supernatant will be stored at -20 ºC until further use.

### Electrophoresis and Western Blotting

Protein content of the oocyte extract will be determined by Lowry et al., (1951) using bovine serum albumin as standard. Protein samples will be resolved in 15% SDS-PAGE (Laemmli, 1970), transferred to PVDF membrane (GE Healthcare) and immunoblotted with appropriate antibodies following standard protocol as described earlier (Khan and Maitra, 2013; Maitra et al., 2014; Das et al., 2013; 2016).

**Total RNA Isolation and Reverse Transcription**

Total RNA will be extracted from cultured oocytes, follicular cells and liver tissues using Tri-Reagent (Invitrogen) according to the manufacturer's protocol. Briefly, 80-100 mg of the tissue will be isolated and homogenized in 1 ml Tri-Reagent. Chloroform (200 µl) will then be added, mixed and centrifuged at 12,000 x g for 10min at 4 ˚C. After centrifugation, the aqueous layer is to be transferred to a fresh tube and mixed well with 500 µl isopropanol to precipitate the RNA. The precipitated RNA will be re-suspended in Nuclease free water and stored at -80 ˚C till further use. The isolated RNA will be subjected to reverse transcription using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas) as per manufacturer's protocol using equal amounts of RNA (1µg).

**Polymerase Chain Reaction (Semi- or Real-time quantitative PCR)**

For both target and reference genes, RT-PCR will be performed in 12.5µl volume containing 2x PCR Master Mix (Fermentas), 10pg-1µg cDNA, 0.1-1µM forward and reverse primers each. PCR would be run on Veriti Thermal Cycler (Applied Biosystems). Real-time quantitative PCR will be set up in Quant Studio 5 PCR system (Thermo Scientifics) with reaction mixture (10 µl) containing 1 µl sample cDNA template diluted (1:10) with nuclease-free water, forward and reverse primers (250 nM of each) and SYBR Green PCR Master Mix (SYBR Green Dye, dNTPs, passive reference I, AmpliTaq Gold DNA Polymerase; Applied Biosystems) and conditions will be optimized as per standard protocols.

**Keywords**

Oocyte; Follicular layer; Theca; Granulosa; Growth factors; IGF; IGFR; EGF; EGFR; Endocrine disrupting chemicals; Maturational competence

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